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A Synopsis on

OVERPRODUCTION OF BIOETHANOL FROM CELLULOSIC WASTES

For

Partial fulfilment of Ph. D. Degree in Biosciences

MODY UNIVERSITY
OF SCIENCE AND TECHNOLOGY
LAKSHMANGARH • RAJASTHAN
A Leading Women's University

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OUTLINE OF THE PROPOSED RESEARCH WORK

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2. Enrolment No.	160647
3. Title of the Research	Overproduction of bioethanol from cellulosic wastes
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b) Geographical Area of Investigation. If any	Not applicable

6. IMPORTANCE OF PROPOSED INVESTIGATION

Ethanol or 'ethyl alcohol' has become a part of our day-today lives especially in transportation sector. It is commonly produced from biomass such as corn, sugarcane, sweet sorghum, starch based substrates, switchgrass, etc. However, this has a very huge impact on agriculture. Due to the demand of these crops, cultivated area under these crops is increasing and performing monoculture every year leads to soil infertility. Because of its commercial application, more crops are diverted to biofuel production instead of food production. This has raised various social and ethical concerns whether the food crops should be used for commercial applications instead of food applications.

One of the positive aspects of producing ethanol is to produce from cellulosic waste materials such as industrial wastes, crop residues, wood residues, urban wastes, forest materials, etc. If such wastes are used as a substrate for bioethanol production, ample quantity of raw material will be available. Furthermore, it could also avoid the problems regarding global climate change, dependence on foreign countries and transportation which is unneeded because trees and grasses can grow anywhere temperate. Ethanol produced from cellulosic wastes will reduce the cost of production and will be a great advantage to the oil companies in producing the blended petrol at a cheaper price.

Hence, cellulosic ethanol which are produced commercially is viewed as a next level of development for biofuel industry.

7. OBJECTIVES OF THE PROPOSED STUDY

- To use cellulosic wastes as a raw material for the production of bioethanol.
- To optimize the pre-treatment method for the cellulosic wastes.
- To isolate/procure bioethanol producing microorganisms.
- Morphological, biochemical and molecular characterization of the potential isolates.
- To check the extent of ethanol tolerance in different isolates/mutants/genetically engineered microorganisms.
- To create physical & chemical mutagens of the potential isolate for over-production of bioethanol.
- Optimization of culture conditions for increase level of bioethanol production.

- Bioethanol extraction and quantification.

8. REVIEW LITERATURE

Ethanol can be produced from various substrates such as lignocelluloses, starch and wastes **[1]**. Lignocellulosic biomass is more favourable than sugar-based crops and starch since it does not compete with food. Further, it takes care of agricultural residues which are meant for burning **[2, 3]**. Lignocellulosic biomass is primarily composed of cellulose (35-50%), hemicelluloses (15-30%), pectin (2-5%) and lignin (12-25%) **[4]**.

For cost-effective lignocelluloses conversion, one of the major challenges is the availability of robust microorganisms which are able to convert all sugars in lignocellulosic hydrolysates efficiently **[5]**. Sugars can be released from lignocellulose by pre-treatment and enzymatic hydrolysis steps **[6]**. Alkaline hydrogen peroxide (AHP) treatment has shown to be effective during treatment of lignocellulosic feedstocks which include grasses, agricultural residues, wood, etc. **[7]**. Pre-treatment process is required to reduce the size of the sample, breakdown the hemicelluloses to sugars and open up the structure of the cellulose component. The portion that contains cellulose is hydrolysed by enzymes or acids into glucose sugar and is fermented to bioethanol **[8]**.

Various factors which influence the production of bioethanol includes pH, temperature, fermentation time, inoculums size, sugar concentration, etc. **[9]**. Stressful conditions like an increase in osmotic stress, ethanol concentration and bacterial contamination are some reasons why yeast cannot survive during fermentation **[10]**. The accumulation of organic acids was also found to contribute to ethanol toxicity **[11]**. For ethanol purification, distillation method is used and is the most dominant and recognised purification technique **[12]**.

Saccharomyces cerevisiae is regarded superior to various filamentous fungi, other yeasts and bacteria in various physiological characteristics for ethanol production **[13]**. It tolerates a wide range of pH **[14]** with acidic pH as optimum **[15]**. It can also tolerate ethanol better than other ethanol producers **[16]**. The immobilization of cells of xylose-fermenting yeasts led to improved ethanol yields **[17]**. Immobilizing yeasts reduce risk of contamination. So, it has been considered as potential alternative for enhancing productivity **[18, 19]**. It makes the separation of cell mass from the bulk liquid easy **[19]** and retain stability of cell activities **[20]**. minimization of production costs **[21, 22, 23]** and reduce fermentation time **[24]**.

Metabolic engineering is enhancing the capabilities of microorganisms by modifying traits such as tolerance to ethanol and inhibitors, hydrolysis of cellulose or hemicelluloses and improvement of sugars transport **[25]**. Besides *Saccharomyces cerevisiae*, *Zymomonas mobilis* is also noteworthy for its high growth rate and high specific ethanol production **[26]**. The transfer of *pdc and adh II* genes which are ethanol fermenting genes from *Zymomonas mobilis* to cellulolytic bacteria could improve the ethanol productivity by converting pyruvate completely to ethanol **[27]**. Hence, the major goal for biofuel production is to develop organisms that utilizes all the major components of lignocelluloses and to produce more gasoline like fuel **[28]**.

9. MATERIALS AND METHODOLOGY:

a) COLLECTION OF SUBSTRATES

Feedstock is one of the most significant cost element in ethanol production. Agro-industrial biomass comprising of lignocellulosic wastes is an inexpensive, abundant and renewable resource which can convert into high value products **[29]**. Substrates such as lignocellulosic biomass which includes agricultural residues like sugarcane baggase, crop residues, corn stover, etc. can act as a promising feedstock.

b) OPTIMIZATION OF THE CELLULOSIC WASTES FOR THE PRE-TREATMENT METHOD

Pre-treatment is one of the most expensive steps during ethanol production due to the use of chemical products and steam. The pre-treatment goal is to break the lignin and to disrupt the crystalline nature of cellulose **[30]**. It is believed that with the advancement of pre-treatment technologies, there would be improvement of efficiency and lowering the costs. Pre-treatment of lignocellulosic residues is necessary because hydrolysis of non-pretreated materials is slow and results in low product yield. Various pre-treatment methods have been used and can be categorized into 3 main groups: Physical (Steam explosion, milling, etc.); Chemical (acid or alkali) and biological pre-treatment (by micro-organisms).

c) ISOLATION OF MICROBIAL STRAINS

The major goal for the production of ethanol is to develop robust organisms that can utilize major lignocelluloses. With the help of metabolic engineering, traits of microorganisms can be modified to enhance high ethanol productivity.

d) **IDENTIFICATION OF SELECTED YEAST ISOLATES ON THE BASIS OF BIOCHEMICAL CHARACTERISTICS**

Biochemical tests are among the most important method for the identification of microbes. It shortens the time, reduce costs and ensure the accuracy of identifying unknown sample. Various tests like carbohydrate fermentation test, catalase test, gelatinase test, amylase test, hydrogen sulfide test, urease test, flocculation test, etc. could be performed.

e) **MOLECULAR CHARACTERIZATION**

- DNA isolation, purification, quantitative and qualitatve analysis.
- 16SrRNA for bacteria sequencing and 18SrRNA sequencing for yeasts.
- ITS region amplification

f) **TO CHECK THE EXTENT OF ETHANOL TOLERANCE IN DIFFERENT ISOLATES/MUTANTS/GENETICALLY ENGINEERED ORGANISMS.**

Microorganisms are sensitive to higher ethanol concentrations. Better understanding of the cellular consequences of ethanol stress defence mechanisms is very important for improving the performance of the strains during stress **[31].** Tolerance levels between 6% to 22% can be checked.

g) **IMPROVING STRAINS THROUGH MUTAGENESIS FOR THE OVER-PRODUCTION OF BIOETHANOL.**

There are various strategies that can be developed to provide best strains for specific industrial goals. Techniques such as mutagenesis or genetic modification strategies to modify targeted traits can generate optimized microorganisms. Induction of random mutations by chemical or physical mutagens will be applied for strain improvement. Mutagenesis, in recent studies is often the initial step for generating genetic variation **[32].**

h) CULTURE CONDITION OPTIMIZATION

Optimization is the suitable condition which can be increased by optimizing culture conditions. It involves suitable conditions of incubation period, temperature, pH, suitable carbon sources as well as salt tolerance. In short, it refers to provide the best condition for the growth of the isolates **[33].**

i) BIOETHANOL EXTRACTION AND QUANTIFICATION.

Distillation method is mainly used for ethanol purification in the industry and is the most recognized and dominant purification technique. It involves taking the fermented ethanol from the water mixture by giving heat to separate them. However, Distillation is a repetition of vaporization and condensation. Therefore, it costs a lot **[34].**

Preparation of potassium dichromate solution and making the standard curve by collecting commercial ethanol sample should be done. Estimation of bioethanol is done by using ethanol sample collected by distillation process. Absorbance at 600 nm should be measured and compare with the standard curve.

10. SIGNIFICANCE AND FUTURE ASPECTS OF WORK.

i. The utmost significance of this work lies in isolating novel strains for ethanol production at industrial level as microbes are used to covert glucose into sugar which later on ferment to bioethanol.

ii. The high cost of extraction is the main problem. By reducing cost of conversion, increasing yields and diversity of crop used, production process can become more efficient.

iii. Increased production of bioethanol can significantly reduce the use of petroleum based products and save for future generations.

iv. This project is important to the society as it will reduce pollution in the environment by utilizing the cellulosic wastes materials which was meant for burning.

v. Second generation of bioethanol (SGB) appears to be bright as the need for renewable energy sources to replace dependence on foreign oil is in high demand.

vi. Countries like India, China, Australia, Europe and some South Asian countries are expanding rapidly their biofuels production and use.

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